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(54) Title: METHOD AND MATERIALS FOR THE IDENTIFICATION OF LIPOPOLYSACCHARIDE PRODUCING MICROORGANISMS

(57) Abstract

Means for the immunological detection of an entire class of microorganisms in clinical samples. The detection is accomplished by reaction of the clinical sample with a class-specific immunological reagent. This reagent is an antiserum either monoclonal or polyclonal in nature, and the detection is based upon reaction of the antiserum with an antigenic determinant which is shared among all members of the detectable class of microorganisms. The presence of the resulting immunological reaction product (e.g. the antigen-antibody complex) may be detected by well-known immunological d tection-systems.

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METHOD AND MATERIALS FOR THE IDENTIFICATION OF LIPOPOLYSACCHARIDE PRODUCING MICROORGANISMS

Field of the Invention

This invention relates to the immunological detection of microorganisms. More specifically, the invention relates to methods and materials useful for the detection of a diverse group of microorganisms in a clinical sample.

Background of the Invention

Microorganisms possessing a cell-surface component known as an endotoxic lipopolysaccharide (LPS) have been identified as the etiologic agents in a wide variety of diseases. Of particular interest are strains of the genera Neisseria, and Chlamydia, which are associated with certain venereal diseases; the incidence of these diseases having reached almost epidemic proportions.

The LPS molecule has been the subject of intense study. See for example, Westphal et al., Bacterial Lipopolysaccharides, Methods of Carbohydrate Chemistry, Vol. V, Academic Press, (1965), pgs. 83-91; and Galanos et. al., Biochemistry of Lipids II, T.W. Goodwin (Ed), Univ. Park Press, (1977), Vol. 14, at page 239.

The structure of the lipopolysaccharide has been described in studies of Gram-negative bacteria such as, Escherichia coli and Salmonella typhimurium. In general, the antigen can be visualized as possessing three component regions. Proximal to and imbedded into the outer portion of the cell membrane is the so-called lipid A component. This component has been associated with the endotoxin properties of the molecule and is believed to be a highly conserved sequence; that is to say, a wide variety of organisms would

likely possess identical lipid A regions. The middle region of the LPS molecule is another conserved region, the so-called core oligosaccharide. The core oligosaccharide is bipartite in structure based upon the types of sugar moieties of which it is composed. The innermost region which is adjacent to the lipid A component contains the unusual sugar, 3-deoxy-manno-octulosonic acid, also known as ketodeoxyoctanoic acid (KDO). The outermost region of the core structure is comprised of a region of seven-carbon sugars (heptoses) which is followed distally by a region containing six-carbon sugars (hexoses). The most distal region of the LPS molecule is the so-called somatic or (0) region comprised of highly-variable polysaccharide components.

It is this outermost region which induces the strongest immune response in infected organisms and it is to this region that antisera produced thereby is directed. Thus, even though a number of different taxa may possess the same Lipid A and core components of the LPS molecules, because of the antigenic variability of the somatic region, antisera produced thereto will be specific for each type of inducing organism.

Microorganisms can thus be classified into chemotypes based upon their carbohydrate composition in the 0 region of the LPS. One method is described by Merrick in U.S. Patent 3,891,508. Such a composition analysis although somewhat useful for purposes of identification is limited by not being able to discriminate between two organisms which may possess the same kinds of sugars but in different proportions or in different sequences.

The LPS molecule when used as an immunogen, may produce certain toxic side-effects due to the biological activity of the molecule. Several patents, such as U.S. Patents 4,185,090 and 4,057,685 relate to methods of reducing the toxicity of the molecule but retaining the antigenicity of the O region. The specificity of the O region in permitting the identification, of only a single species is disclosed by Wallace et. al. in U.S. Patent 4,115,543 relating to the identification of Neisseria gonorrhoeae. Other approaches attempt to find other antigenic inducers and eliminate the LPS molecule from the immunizing mixture as for example, described by Ayme in U.S. Patent 4,337,243.

It has been recently demonstrated that conventional polyclonal sera raised to Salmonella typhimuruin Re mutants cross-react with various Chlamydia species (Nurminen, et al., Science 220: 1279-81 (1983)). There is no suggestion of the diagnostic utility nor of the wider reactive potential of the antibodies as produced and described hereinbelow.

Human monoclonals of the IgG_1 subclass have been demonstrated to be reactive with a limited class of microorganisms namely members of the Chlamydia genera. Although, not particularly well-characterized, the antigen to which the monoclonals are directed is referred to as a lipoprotein complex. Thus, it is probably closely related to the Chlamydia group specific protein as characterized by Caldwell, et al., (J. Immuno. 115: 963-68 (1975)) and clearly distinct from the endotoxic-glycolipid as described herein.

In an interesting, but unrelated application, (PCT application WO80/01109) the LPS molecule itself has been used as a marker reagent; being chemically attached to a ligand,

it permits the detection of the LPS-ligand complex by standard LPS assay method (The Limulus amebocyte assay).

None of the above references relate to the object of the subject invention, that being, the generation of immunological reagents useful in the identification of a diverse group of LPS producing microorganisms.

Brief Description of the Invention

The subject invention provides a means for the immunological detection of an entire class of microorganisms in clinical samples. The detection is accomplished by reaction of the clinical sample with a class-specific immunological reagent. This reagent is an antiserum either monoclonal or polyclonal in nature, and the detection is based upon reaction of the antiserum with an antigenic determinant which is shared among all members of the detectable class of microorganisms. The presence of the resulting immunological reaction product (e.g. the antigen-antibody complex) may be detected by well-known immunological detection systems employing analytically indicatable reagents.

The subject invention is particularly useful for the detection of a class of microorganisms which produce endotoxin or endotoxin-like molecules. The shared antigen determinant in this case, is often a component of a cell-surface lipopolysaccharide. Microorganisms which display this antigen and thus are members of a detectable group comprise Chlamydia, Rickettsia, and other gram-negative microorganisms such as Neisseria, Brucella, Escherichia, Salmonella and the like.

In a preferred embodiment, an antibody to the shared determinant of the detectable class is used as the "capture" antibody in a differential screening assay system.

Once recovered from a clinical sample as a member of the reactive class of microbes which possess the shared antigen, the captured cells or antigens are then subsequently reacted with more specific antisera to indicate which particular genera, species or serotype of microorganism is present.

For example, a first antibody is synthesized which reacts with the "gram-negative-like" portion of the endotoxic glycolipid, and second antibody is synthesized which reacts with a Chlamydial genus specific determinant. The first antibody can be used alone to detect all gram-negative-like microorganism by simple fluorescent or agglutination assays. Alternatively, the two antibodies may be used in combination in a solid phase immunoassay to discriminate the Chlamydia genera from among the other members of the reactive class.

In a further embodiment, the Chlamydia genus specific antibody may be used alone to detect all members of the Chvamdia genus or as the capture antibody in a differential screening assay whereby the Chlamydia genus specific antibody is used to react with all members of the genus and a second antibody or antibodies which are reactive to Chlamydia species determinants are employed to differentiate each species in the reactive genera..

The subject invention provides an immunological reagent for the detection of shared antigenic determinants of at least two microorganisms comprising an antibody specifically reactive to said shared determinants, more specifically an immunological reagent for the detection of two or more endotoxin or endotoxin-like lipopolysaccharide producing microorganisms comprising a monoclonal antibody specifically reactive with shared antigenic determinants of said lipopolysaccharide.

Also provided are methods for the immunological detection of an antigen shared by a plurality of taxonomically distinct microorganisms. One such method comprises: contacting a sample containing said microorganisms or antigens thereof with an analytically indicatable antibody to form an immunocomplex of said microorganism or antigen thereof and said antibody, said antibody being characterized as having affinity for an antigenic determinant shared by said microorganism. There is also provided a two-site immunometric assay to identify an antigen in a sample comprising forming a ternary complex of a first labelled antibody, said antigen, and a second antibody, said second antibody being bound to a solid carrier insoluble under ternary complex forming conditions wherein the presence of the antigen is detected and identified by measuring either the amount of labelled antibody bound to said solid carrier or the amount of unreacted labelled antibody, in which the improvement comprises employing as said second antibody a monoclonal antibody characterized in having affinity for an antigenic determinant site of said antigen which is shared by a plurality of taxonomically distinct microorganisms and employing as a first antibody an antibody characterized as having an affinity for an antigenic determinant site specific to a taxonomically distinct microorganism.

Detailed Description of the Invention

This invention provides reagents and methods for the detection and subsequent differential identification of an entire class of microbes. For the purposes of this description, class is not used in its strict biological/taxonomic meaning but rather in a more general sense referring to a group of microorganisms that express a

common antigenic determinant. The terms antigenic determinant, antigenic determinant site (ADS) or epitope as used throughout, refer to a limited, specific part of an antigen which is the inducer of antibody formation and which is the region of the antigen to which the induced antibody reacts. The number of antigenic determinant sites possessed by a given antigen will vary and may range from 1 to 10 or 20 or more depending upon the size and immunological complexity of the antigen molecule. Those antigenic determinants which are unique to a given antigen are known as specific determinants and are useful in discriminating between two different types of antigen molecules. Those determinants which are present on two or more antigens are known as shared determinants and, as mentioned above, form the basis of an immunological class of antigens.

An antigen of particular interest with respect to the subject invention, as described above, is the lipopolysaccharide (LPS)/glycolipid antigen associated with endotoxin or endotoxin-like molecules produced by Gram-negative microorganisms.

With reference to the discussion of antigenic determinants above, each of the three regions of the LPS/glycolipid molecule could be considered to be comprised of one or more separate ADSs. The somatic region is considered to be a specific determinant since, because of its high variability, antibodies raised to the somatic region of one LPS would not likely cross-react with another O determinant; thus organisms expressing a particular LPS may be distinguished from others by virtue of the somatic ("body") types.

In contrast, because of the conserved nature of the Lipid A or core region, different organisms that possess endotoxin or endotoxin-like entities will have antigenetically similar regions. Thus, antibodies raised to these determinants will react with an entire class (group) of microorganisms, those of which can express LPS regardless of somatic type.

In Nature, the induction of anti-Lipid A or anti-core poly- saccharide reactive antibodies does not occur readily because of the existence of the somatic determinants attached thereto. However, there are known to exist in Salmonella mutations which affect various steps in the synthesis of the LPS/glycolipid molecule. Certain of these mutations result in strains which produce truncated forms of the LPS/glycolipid molecule and are known as R mutants. One of the most defective R types known is designated as Re. This mutant is well-known having been described by Stocker, et al. (J. Gen'l. Microbiol., 70: 527 (1972)) and is publically available from the Salmonella Stock Center, Dept. of Biology, University of Calgary, Calgary, Alberta, Canada.

As described herein it is possible to prepare antibodies which react specifically with the Lipid A or core-determinants thus providing valuable immunological reagents for the detection of a class of microorganism, i.e., those microorganisms expressing LPS/glycolipid regardless of somatic type.

A variety of protocols may be used to provide the Lipid A or core-polysaccharide reactive antibodies. Conventional immunization and isolation procedures result in

the production and recovery of polyclonal antisera directed against the shared determinants of the LPS antigen. Alternatively, somatic cell hybridization procedures may be employed to construct hybridoma cell lines capable of generating monoclonal antibody to the determinants in question. These procedures are more fully described in the Examples which follow.

Although it has been recently demonstrated that conventional polyclonal sera raised to Salmonella typhimurium Re mutants cross-react with various Chlamydia species, (Nurminen, et al., Science 220: 1279-81 (1983)), there is no suggestion of the diagnostic utility nor of the wider reactive potential of the antibodies as produced and described herein.

It has been surprisingly discovered that the immunological reagents described herein not only react with Gram-negative bacteria expressing LPS such as Salmonella and Neisseria, but also all Chlamydia species as well. Predicated on this finding it is now possible to generate immunological reagents of extremely broad specificty such as a single antibody reactive with any and all LPS/glycolipid producing organism. Appropriate target organisms include Gram-reactive bacteria such as Salmonella, Brucella, Escherichia, Neisseria, Serratia, Pasteurella, Proteus, Shigella, Klebsiella, as well as Chlamydia, and Rickettsia.

Furthermore a group of immunological reagents of intermediate specificity are also disclosed herein. Antibodies of the group react specifically with members of all species within a given genera but not with organisms outside that genera. These antibodies are said to be genera-specific.

Thus, by using an antibody of broad specificity, it is possible to perform a single test for a large number of LPS producing organisms merely by reacting said antibody with a clinical sample and detecting any antibody-antigen reaction which occurred by measuring the antigen and antibody complex directly, conventionally by monitoring an analytically indicatable detection system.

Thus antibodies of the subject invention can be used directly and can be modified as necessary to render them useful in any of the well-known immunological detection systems. Although not meant to be exhaustive, suitable methods for detection of the antigen-antibody reaction include: precipitation, agglutination, double antibody techniques, fluoroscent-, enzyme-, ferritin-, or radioactively-labelled antibodies ^{125}I-/or similarly radioactively labelled antibodies, protein A from Staphylococcus aureus, enzyme-linked immunoassay (ELISA) or biotin-based assay systems. The subject invention provides specific reagents for the reaction with the LPS/glycolipid antigen and not any particular method of indicating the resulting reaction product. The most appropriate method of indication can be determined by one skilled in the art. A wide variety of indicator systems are contemplated, and the subject invention should not necessarily be limited by a particular method of indicating the positive reaction.

In a preferred embodiment, the immunological reagent (the anti-Lipid or anti-core LPS reactive antibody of or monoclonal origin) is employed as a capture antibody in a "sandwich-type" assay as described in U.S. Patent 4,376,110. According to this embodiment, the anti-Lipid A or

core-reactive antibody is attached to a solid support by widely known cross-linking methods such as described by S. Avrameas In: Immunoassays for the 80's Voller et al. (Eds) University Park Press, Baltimore (1981) at page 85.

A clinical sample, which may be defined as body fluids or secretions such as blood, serum, saliva, stool, urine, milk, topical washing of skin or genitals, tissue samples or homogenates thereof and samples of culture fluids of infected cells or homogenates thereof, is reacted with the supported immunological reagent under conditions which promote the formation of an immune complex between said supported reagent and any material containing the Lipid A or core determinants, thereby binding to the support one or more members of a variety of taxonomic groups of micro-organisms. The specific identity of the constituent members of the bound population may be determined, after washing the initial reaction product to remove unbound contaminants, by subsequently reacting the bound population with specific antisera for each specific taxonomic type of interest e.g. genera-specific, species-specific or serotype-specific antisera. An advantage employing a reagent of broad specificty is that considerable research and development as well as production time and expense is saved by eliminating the necessity of generating a large number of reagents of narrower specificity to accomplish the same result.

In a further embodiment, the above described "sandwich" assay can be carried out by providing a plurality of separate reactions of the supported "capture" reagent followed by detection of the reacted sample by adding a particular genera-, species- or serotype-specific reagent to

an individual reaction or alternatively two or more specific reagents may be added to a single reaction. Thus it is possible to detect simultaneously in one reaction two or more taxomonically distinct microorganisms. In this later variation of the assay, if the clinical sample contains both Chlamydia as well as Neisseria both organisms would be retrieved by the "capture" antibody attached to the solid support. Once bound, addition of two additional types of detectably labelled antibodies, one specific for Chlamydia and the other specific for Neisseria would permit the simultaneous detection of both organisms. Detection is facilitated by employing analytically distinct reagents is labelling the specific antibody. For example, the Chlamydia specific antibody may be labelled with B-galactosedase and the product of the ELISA reaction measure of one spectrophotometric wavelength whereas the Neisseria specific antibody labelled with alkaline phosphatase and the ELISA reaction product measured at a second wavelength. Other variations employ colorimetric blending of two separate reaction products or the use of a radiometric/photospectrometric combination are also possible. The following examples further illustrate various facets of the invention but are not to be viewed as limitations of the invention per se.

Example 1

Methods for the Production and Recovery of Polyclonal Antibody to Endotoxic Glycolipids

New Zealand white rabbits weighing approximately 2 kg and 8 to 10-week old BALB/c mice were injected subcutaneously with an emulsion containing equal parts of incomplete Freund's adjuvant and water in which was suspended formalin-fixed Salmonella typhimurium Re chemotype whole cells. Lyophilized whole cells were formalin fixed by suspension in 0.2% formaldehyde and incubation overnight at 25°C. They were diluted into water used for preparation of the emulsion for injection so that each rabbit received 1.0 mg and each mouse, 0.1 mg. The animals were injected subsequently with the same quantities of whole cells suspended in water and given intravenously at 2 to 6-week intervals for 3 to 6 months. The animals were anesthetized and exanquinated by cardiac puncture. Sera collected from the blood was stored frozen or filtered through 0.45 um filters and stored at 4°C in 0.1% sodium azide. This antisera is termed anti-Re glycopid antisera.

Other rabbits and mice were immunized in a similar scheme but with whole cells treated with 1% acetic acid in water at 100°C for 2 hr (Osborn, M.J., Proc. Nat. Acad. Sci. U.S.A. 50, 499 (1963) and coated with Lipid A. The acid treatment removes the KDO and core saccharides from endotoxin in the walls of the bacteria, leaving the lipid A region intact. Purified endotoxin extracted from lyophilized whole bacteria by the procedure of Galanos et al. (Eur. J. Biochem. 9, 245 (1969)) was also converted to Lipid A by treatment

with acetic acid. This lipid A was adsorbed to the acetic acid-treated whole bacteria by mixing into a homogeneous suspension the lipid A and the acid-treated bacteria and then evaporating the water under reduced pressure at 50-60°C until a thick slurry was generated.

Animals immunized with this antigenic preparation produced a sera termed anti-lipid A glycopid antisera. In certain circumstances, some of the animals received an equal mixture of untreated whole cells and acid-treated, lipid A-coated whole cells. Sera from these mice is termed anti-Re-Lipid A glycolipid antisera.

These antisera were tested in an enzyme immunoassay for reactivity to purified endotoxic glycolipids (termed Re endotoxin or lipid A) prepared as described above. These glycolipids were attached to polystyrene plates by incubating 0.1 ml/well of a solution containing endotoxic glycolipid (25 ug/ml), triethylamine (0.5 ul/ml), sodium azide (0.15) and $MgCl_2$ (1 mM) for 6 to 18 hours at 37°C. This solution was removed and replaced with phosphate buffered saline (pH 7.4) containing 1 mM MgCl. The plates were stored at 4°C. The test antisera or normal rabbit sera or normal mouse sera at various dilutions (commonly 1/1000) was added to each well (100 ul/well after removal of the saline solution used for storage). The plates were incubated 3 to 18 hours at 25 or 37°C; the sera was decanted; the plates were washed with buffered saline containing MgCl (three times, 200 ul each time); alkaline phophatase-conjugated antiserum to rabbit or mouse immunoglobulin (IgG and IgM) (purchased from Tago Inc., Burlingame, CA) was added and incubated for 6 to 18 hours at 37°C. The plates were again washed as before and 100 ul of

alkaline phosphatase substrate p-nitro-phenol phosphate in 1% diethylamine and 1 mM MgCl was added. The plates were examined visually for yellow color development or spectrophotometrically at 405 nm following incubation at 25°C for ½-3 hours.

Example 2

Antibody to Endotoxins of Chlamydia Species and Neiserria Species

Rabbits and mice were immunized with formalin-fixed elementary bodies of C. trachomatis (strain L2/434/Bu(L2) grown in HeLa cells (see Caldwell et al. Infect. Immun. 31, 1161 (1981) for preparation of purified elementary bodies). The formalin fixation and immunization scheme was as described in Example 1 above. Other animals were immunized with formalin-fixed N. gonorrhea (laboratory strain F62 phenotyped $P^{++}O^{+}$) in a similar immunization protocol.

Sera from these animals was tested for antibody reactivity in an immunoassay as described above in Example 1. Whole elementary bodies or endotoxic extracts C. trachomatis or whole bacteria N. gonorrhea were attached to polystyrene plates (10^7 organisms or equivalent extract per ml of phosphate buffered saline; 0.15 ml per well). The endotoxic extracts were obtained as described in Example 1. The plates were subsequently treated with MEM solution containing 10% fetal calf serum and 0.25% bovine serum albumin (0.25 ml per well). Some of the wells were treated with sodium metaperiodate (50 mM) in sodium acetate (50 mM, pH 5.6 for 3 hours at 37°C. This treatment destroys the immunological reactivity of certain sugars in the endotoxins or endotoxic glycolipids, but does not affect the immunological reactivity of peptides in proteins.

Example 3

Monoclonal Antibody to Endotoxic Glycolipids

A. Female BALB/c mice were immunized with a mixture of untreated and acid-treated cells as described in Example 1. The mice received 4 to 6 injections of immunogen and were euthanized three days after the last injection. The spleens were removed aseptically and homogenized between glass microscope slides. The cells were washed in minimum essential medium (MEM) and the red blood cells lysed by treatment for 45 seconds in sterile water. The cells were diluted into MEM to stop the lytic process. The spleen cells remaining were pelleted at room temperature upon centrifugation for 15 minutes at 1500 rpm in a Beckman TJ-6 table top centrifuge. To this pellet was added 2 to 5 x 10^7 SP2/0 myeloma cells (Institute for Medical Research, Camden, N.J. 08103) that had been washed twice in MEM. Typically spleen cells from one spleen were combined with myeloma cells recovered from vigorously shaking three, 250 cm^2 Falcon tissue culture flasks used for maintenance of the myeloma cell line.

The combined spleen cells and myeloma cells were centrifuged for 10 minutes at 600 rpm in the Beckman TJ-6 centrifuge at room temperature. The pellet was dislodged after decanting the MEM. The cells were then treated for 1 minute with 1.0 ml of polyethylene glycol 4000 (Sigma Chemical Co.) which had been sterilized and diluted with an equal part of MEM just before use. It was important to dilute the polyethylene glycol with MEM while it was still hot from sterilization. It was cooled to 37°C before addition to the spleen cell and myeloma cell pellet. Sequential additions of 1.0, 2.0, and 4.0 ml of MEM followed

at 1.0, 2.0, and 4.0 minute intervals. The fusion treatment was terminated by the addition of 43 ml of MEM with 10% fetal calf serum (Hyclone Laboratories, Sterile Systems, Logan, Utah) and Hypoxanthine, aminopterine, and thymidine (HAT). The cells were distributed into two, 24-well tissue culture trays. Fresh HAT media was added the following day, and twice weekly for two weeks. The cells were cultured an additional two weeks in hypoxantine- and thymidine-containing MEM and fetal calf serum. Wells supporting the growth of hybridomas covering approximately one third the surface area of the well were screened for antibody as described in Example 1 by the enzyme immunoassay procedure.

Generally, at 5 to 8 weeks following the fusion date, cells from positive wells were cloned by limiting dilution. The cells were diluted to 30 cells and 10 cells per ml of media; 0.1 ml of these solutions were dispensed in 96-well trays. An additional 0.1 ml of media containing spleen cells (2.5×10^6/ml) was added; the trays were wrapped in plastic film and incubated at 37°C in a humidified chamber in the presence of 5% CO_2 for 2 to 6 weeks. The wells were again screened for antibody production. A population was considered cloned when obtained from a well that was one of a group of wells receiving the same cell contractation only if that group had growth of hybridomas in one third or fewer wells. Generally clones were cloned 3 to 4 times to ensure single-cell origin.

Positive clones were transferred into 24-well tissue culture plates and after 1 to 3 weeks, into flasks in order to expand the populations. The culture supernatants were collected and stored at 4°C in 0.1% sodium azide. Ascites were collected from BALB/c female mice to 4-8 weeks following being primed with pristine (0.5 ml i.p. 10 to 30 days before injection of hybridoma cells) and injected i.p. with 10^6 to 10^7 hybridoma cells.

B. In a modification of the above procedure, P3X63Ag8Z myeloma cells are substituted for the SP2/0 cells and substantially similar results are obtained.

EXAMPLE 4

Solid Phase Immunometric Assay

The principle of detection of antigens (chlamydial endotoxin or chlamydia in this example) by a solid phase immunometric assay whereby the antigen is sandwiched between two antibodies is well documented (for review see Immunoassays for the 80s, A. Voller, A. Bartlett and D. Bidwell, eds. University Park Press, Baltimore (1981) at page 85. Heterogeneous enzyme immunoassays by S. Avrameas). The general scheme of such an assay is 1) adsorbtion or covalent linking of the first antibody to a solid phase such as a polystyrene or nylon tube, plate or bead, 2) incubation of the test material containing the antigen which will specifically bind to the first antibody, 3) washing away unreacted antigen(s) not specifically bound, 4) addition of the second antibody which will also react with the antigen already bound to the first antibody. (the second antibody may be labelled by iodination with radioactive iodine, e.g., or by being conjugated with an enzyme such as alkaline phosphatase), 5) washing an unreacted labelled second antibody from the reaction vessel, and finally 6) measuring the presence of the labelled antibody remaining in the vessel (radiometrically or enzymatically).

The protocol used herein is after the procedure described by E. Engvall, K. Jonsson, and P. Perlmann (Biochim. Biophys. Acta 251, 427-434 (1971)). In brief the procedure was to coat polystyrene plates with polyclonal or monoclonal antibody to endotoxic glycolipids (see Example 1 and 2 for preparation of these antibodies). The antibody

solutions were diluted in 0.1 M sodium carbonate buffer (pH 10) and 100 ul dispensed into each well of a 96-well polystyrene plate. They were incubated at 37°C for 3 hours minimum (maximum of 18 hours), washed with phosphate buffered saline (pH 7.4) containing 0.9% sodium chloride and 0.05% Tween-20 and 0.5% bovine serum albumin, the test material containing chlamydia or endotoxic glycolipid antigens diluted in the same type solution as used for the wash step was added (100 ul per well), the plates were incubated a minimum of 3 hours at 37°C, washed as before (200 ul per well per wash, 4 washes at 3 minute intervals), the second labelled antibody (immunoglobulin conjugated to alkaline phosphatase by the procedure described by S. Avrameas (Immunochemistry 6, 43 (1969)) was added and incubated 3-18 hours at 37°C, the reaction vessels were washed as before, 100 ul of substrate solution containing p-nitro-phenol phosphate (Sigma Chemical Col., St. Louis, MO) (1 mg/ml) in 1% diethylamine and 1 mM $MgCl_2$ was added. The reaction was read visually after development of a distinct yellow color in positive reaction wells or quantified spectrophotometrically at 405 nm in a Beckman DU-8 spectrophotometer.

EXAMPLE 5

Antibody Reactivity in Enzyme Immunoassay

Protocol: The various antigens were coated on the surfaces of polystyrene plates as described in Example 1. The antisera and monoclonal antibody solutions were added and processed as described. The reactivity of these antibodies to the antigens were detected using alkaline phosphatase conjugated to anti-mouse or anti-rabbit immunoglobulins.

TABLE I

Source of Antibody	Antigen coated on wells						
	Not treated					Periodate treated	
A. Polyclonal	None	LA	Re	GL	N.g.	C.t.	N.g.	C.t.
Rabbit anti-Re GL	-	-	++	±	++	-	±
Rabbit anti-LA	-	++	±	±	+	+	+
Mouse anti-Re-LA GL	-	++	++	±	++	+	++
Mouse anti-C. trachomatis	-	-	-	-	++	-	+
Mouse anti-N. gonorrhoeae	-	-	-	++	-	+	-
Normal Rabbit sera	-	-	-	-	-	-	-
Normal Mouse sera	-	-	-	-	-	-	-
B: Monoclonal (mouse)							
Anti-Re GL (clone: Re-1)	-	-	++	±	++	-	-
Re-2)	-	-	++	±	++	-	-
" Re-3)	-	-	++	±	++	-	-
Anti-LA (clone:LA-1)	-	++	±	-	-	-	+
LA-2)	-	++	±	+	+	-	++
LA-3)	-	++	±	-	±	-	+
LA-4)	-	++	+	+	+	++	++
LA-5)	-	++	+	+	+	++	++
Anti-C-GL(clone:C-1)	-	-	-	-	++	-	-
C-2	-	-	-	-	++	-	-
C-3	-	-	-	-	++	-	-
C-4	-	-	-	-	++	-	-
C-5	-	-	-	-	++	-	-

Abbreviations:

1) N.g. = Neiserria gonorrhoeae whole cells
2) C.t. = Chlamydia trachomatis elementary bodies
3) LA = Lipid A
4) Re GL= Re endotoxic glycolipid
5) C-Gl = Chlamydia glycolipid

" -" (negative) = Absorbancy at 405mm below 0.1
6) "+" (positive) = " between 0.31 & 0.5
"++" (") = greater than 0.5
"±" (weakly positive) = between 0.1 & 0.30

From the above data it can be seen that both polyclonal and monoclonal antisera can be generated that will react with endotoxic glycolipids from a variety of sources. Both anti-Re glycolipid antisera and anti-Lipid A antisera reacts with periodate-sensitive determinates in C. trachomatis elementary bodies. Weak reactivity of these antisera is seen to N. gonorrhoeae. The periodate-sensitivity of certain of these reactions is evidence that the determinate recognized by these antisera is the endotoxic glycolipid present in the cell walls of the microbes. The antisera are defined by their reactivity to purified endotoxic glycolipid and Lipid A from Salmonella whole cells. However, these antisera contain antibodies to to other non-endotoxic molecules in the whole cells such as proteins. Only the anti-endotoxic glycolipid antibodies bind to the chlamydial and gonococcal microbes and this binding is in certain cases prevented by pretreating the microbes with periodate which destroys the unprotected sugars of the endotoxic glycolipids within the whole cells or elementary bodies. However, if the epitope comprises a chemically modified sugar (e. g. amino sugars) the periodate treatment is ineffective.

Monoclonal antibodies with reactivity similar to the polyclonal antisera can also be generated. This reactivity, too, in certain cases is periodate-sensitive, thereby providing further evidence that the epitopes being recognized on the C. trachomatis and N. gonorrhoeae are the sugar moieties of the endotoxic glycolipids in the cell walls or membranes. In addition, these moieties are immunologically identical as defined by monoclonal antibody reactivity to some of the sugar moieties in the endotoxic glycolipids of the core or Lipid A region of gram negative bacteria.

Certain of the clones described in Table I were deposited with the American Type Culbum Collection 12301 Parklawn Drive, Rockville, Maryland on January 19, 1984 and the requisite fees were paid. Access to the cultures will be available during the pendency of the patent application to one determined by the Commissioner to be entitled thereto under 27 CFR §1.14 and 35 USC §122. All restrictions on availability of said culture to the public will be irrevocably removed upon the granting of the instant application and said culture will remain permanently available during the term of said patent. Should the culture beocme nonviable or be inadvertently destroyed, it will be replaced with viable culture(s) of the same taxonomic description.

Each deposited clone was serotyped and assigned the accession number as shown in Table II.

TABLE II

Clone		Antibody Type	Accession No.
Anti-Re GL	Clone Re-1	IgG_3 (K)	HB8482
Anti-Re GL	Clone Re-2	IgG_3 (K)	HB8481
Anti-LA	Clone LA-4	IgM (K)	HB8480
Anti-LA	Clone LA-5	IgM (K)	HB8479
Anti-C GL	Clone C-2	IgG_{2a} (K)	HB8478

WHAT IS CLAIMED IS:

1. An immunological reagent for the detection of shared antigenic determinants of at least two microorganisms comprising an antibody specifically reactive to said shared determinants.

2. The reagent according to Claim 1 wherein said microorganisms are selected from the group consisting of Escherichia, Salmonella, Brucella, Neiserria, Chlamydia, and Rickettsia.

3. The reagent according to Claim 1 wherein said determinant is a portion of a macromolecule.

4. The reagent according to Claim 3 wherein said macromolecule is an endotoxic or endotoxic-like lipopolysaccharide.

5. The reagent according to Claim 3 wherein said portion of a macromolecule comprises Lipid A region or core oligosaccharide region of an endotoxic or endotoxic-like lipopolysaccharide.

6. The reagent according to Claim 5 wherein said region is the Lipid A region.

7. The reagent according to Claim 5 wherein said region is the core oligosaccharide region.

8. The reagent according to Claim 1 wherein said antibody is a monoclonal antibody.

9. An immunological reagent for the detection of two or more endotoxin or endotoxin-like lipopolysaccharide producing microorganisms comprising a monoclonal antibody specifically reactive with shared antigenic determinants of said lipopolysaccharide.

10. The reagent according to Claim 9 wherein said microorganisms are selected from the group consisting of Escherichia, Salmonella, Brucella, Neiserria, Chlamydia, and Rickettsia.

11. The reagent according to Claim 9 wherein said determinants comprises the Lipid A region or core oligosaccharide region of said lipopolysaccharide.

12. The reagent according to Claim 11 wherein said determinant comprises the Lipid A region.

13. A method for the immunological detection of an antigen shared by a plurality of taxonomically distinct microorganisms comprising:

contacting a sample containing said microorganisms or antigens thereof with an analytically indicatable antibody to form an immunocomplex of said microorganism or antigen thereof and said antibody, said antibody being characterized as having affinity for an antigenic determinant shared by said microorganism.

14. The method according to Claim 13 wherein said antibody is analytically detected by fluorometric, enzymatic or radiometric measurement of fluoroscent, enzymatic or radioactive material, chemically complexed with said antibody.

15. The method according to Claim 13 wherein said indicatable antibody is analytically indicated by reaction with a second antibody specific for said indicatable antibody, said reaction being indicated by fluorometric, enzymatic or radiometric measurement of fluoroscent, enzymatic or radioactive material chemically complexed to either of said antibodies.

16. The method according to Claim 13 wherein said shared antigenic determinant is a portion of a cell-surface macromolecule.

17. The method according to Claim 16 wherein said macromolecule is an endotoxic or endotoxic-like lipopolysaccharide.

18. The method according to Claim 16 wherein said-portion of a macromolecule comprises a Lipid A region or core-oligosaccharide region of an endotoxin or endotoxin-like lipopolysaccharide.

19. The method according to Claim 18 wherein said region is the Lipid A region.

20. The method according to Claim 18 wherein said region is the core-oligosaccharide region.

21. The method according to Claim 13 wherein said sample is a clinical sample selected from the group consisting of blood, serum, saliva, stool, urine, milk, topical washings of skin or genitals, tissue samples or homogenates thereof and samples of cell culture fluids of infected cells or homogenates thereof.

22. The method according to Claim 13 wherein said indicatable antibody is a monoclonal antibody.

23. In a two-site immunometric assay to identify an antigen in a sample comprising forming a ternary complex of a first labelled antibody, said antigen, and a second antibody said second antibody being bound to a solid carrier insoluble under ternary complex forming conditions wherein the presence of the antigen is detected and identified by measuring either the amount of labelled antibody bound to said solid carrier or the amount of unreacted labelled antibody: the improvement comprising employing as said

second antibody a monoclonal antibody characterized in having affinity for an antigenic determinant site of said antigen which is shared by a plurality of taxonomically distinct microorganisms and employing as a first antibody an antibody characterized as having an affinity for an antigenic determinant site specific to a taxonomically distinct microorganism.

24. The method according to Claim 23 wherein said antigenic determinant site reactive with said first antibody and said antigenic determinant site reaction with said second antibody are regions of the same macromolecule.

25. The method according to Claim 24 wherein said macromolecule is an endotoxic or endotoxic-like lipopolysaccharide.

26. The method according to Claim 24 wherein said antigenic determinant reaction with said second antibody is comprised of the Lipid A region or core oligosaccharide region of an endotoxic or endotoxic-like lipopolysaccharide.

27. The method according to Claim 26 wherein said region is the Lipid-A region.

28. The method according to Claim 26 wherein said region is the core oligosaccharide.

29. The method according to Claim 24 wherein said antigenic determinant site reaction with said first antibody is comprised of the somatic region of an endotoxic or endotoxic-like lipopolysaccharide.

30. The method according to Claim 24 wherein said antigenic determinant site reactive with said second antibody and said antigenic determinant site reactive with said first antibody are regions of separate macromolecules.

31. The method according to Claim 30 wherein said antigenic determinant site reactive with said second antibody is the Lipid A or core oligosaccharide region of an endotoxic or endotoxic-like lipopolysaccharide.

32. The method according to Claim 30 wherein said antigenic determinant site reactive with said first antibody is a region of any nonendotoxic or endotoxic-like lipopolysaccharide molecule with the proviso that said determinant is specific to a taxonomically distinct microorganism.

33. The method according to Claim 24 wherein said first labelled antibody is labelled with a fluorometric, enzymatic or radioactive material.

34. A monoclonal antibody produced by a hybridoma formed by a fusion of cells from a mouse myeloma and spleen cells from a mouse previously immunized with Salmonella Re endotoxin glycolipid.

35. The monoclonal antibody of Claim 34 which is produced from a hybridoma formed by the fusion of SP2/0 myeloma cells and Balb/c mouse spleen cells.

36. The monoclonal antibody of Claim 34 wherein said hybridoma is selected from the group consisting of Clone Re-1 (ATCC. No. HB 8482) and Re-2 (ATCC. No. 8481).

37. A monoclonal antibody produced by a hybridoma, formed by a fusion of cells from a mouse myeloma and spleen cells from a mouse previously immunized with Lipid A.

38. The monoclonal antibody of Claim 37 which is produced from a hybridoma formed by the fusion of SP2/0 myeloma cells and Balb/c mouse spleen cells.

39. The monoclonal antibody of Claim 37 wherein said hybridoma is selected from the group consisting of Clone LA-4 (Ascc. No. HB8480) and LA-5 (Ascc. No. HB8479).

40. A monoclonal antibody produced by a hybridoma formed the fusion of cells from a mouse myeloma and spleen cells from a mouse previously immunized with Chylamydia glycolipid.

41. The monoclonal antibody of Claim 40 which is produced from a hybridoma formed by the fusion of SP2/0 myeloma cells and Balb/c mouse spleen cells.

42. The monoclonal antibody of Claim 40 wherein said hybridoma is Clone C-2 (ATCC. No. HB 8478).

43. A kit for use in the immunological detection of an antigen shared by a plurality of taxonomically distinct microorganisms comprising antigen reactive means comprising an antibody specifically reactive with said antigen, said antibody being further characterized in being chemically complexed with an analytically detectable reagent.

44. The kit of Claim 43 wherein said reagent comprises fluoroscent, enzymic or radioactive material.

45. The kit of Claim 44 wherein said reagent is the enzyme alkaline phosphatase.

46. The kit of Claim 43 wherein said shared antigen is an endotoxic or endotoxic-like lipopolysaccharide.

47. The kit of Claim 43 wherein said antibody is the monoclonal antibody of Claim 34.

48. The kit of Claim 43 where said antibody is the monoclonal antibody of Claim 37.

49. The kit of Claim 43 wherein said antibody is the monoclonal antibody of Claim 40.

50. A kit for use in the immunological detection of two or more endotoxin or endotoxin-like lipopolysaccharide producing microorganisms comprising:

1) a lipopolysaccharide reactive means comprising a first antibody specifically reactive with said lipopolysaccharide, and

2) a second antibody specifically reactive with said first antibody, said second antibody being further characterized in having chemically complexed thereto an analytically detectable reagent.

51. The kit of Claim 50 wherein said reagent comprises fluoroscent, enzymic, or radioactive material.

52. The kit of Claim 51 wherein said reagent is the enzymic alkaline phosphatase.

53. The kit of Claim 50 wherein said first antibody is the monoclonal antibody of Claim 34.

54. The kit of Claim 50 wherein said first antibody is the monoclonal antibody of Claim 37.

55. The kit of Claim 50 wherein said first antibody is the monoclonal antibody of Claim 40.

56. A kit for use in a two-site immunometric assay for the identification of a plurality of taxonomically distinct lipopolysaccharide producing microorganisms comprising:

1) a first lipopolysaccharide reactive means comprising a first antibody specifically reactive with an antigenic determinant of said lipopolysaccharide said determinant being specific to a taxomically distinct microorganism, said first antibody being further characterized in having chemically complexed thereto an analytically detectable reagent, and

2) a second lipopolysaccharide detecting means comprising a second antibody specifically reactive to an antigenic determinant of said lipopolysaccharide, said determinant being shared by a plurality of lipopolysaccharide producing microorganisms said second antibody being further characterized in being bound to a solid carrier.

57. The kit of Claim 56 wherein said reagent comprises fluoroscent, enzymic, or radioactive material.

58. The kit of Claim 57 wherein said reagent is the the enzyme alkaline phosphatase.

59. The kit of Claim 56 wherein said microorganisms are selected from the group consisting of Escherichia, Salmonella, Brucella, Neiserria, Chlamydia, and Rickettsia.

60. The kit of Claim 56 wherein said second antibody is the monoclonal antibody of Claim 34.

61. The kit of Claim 56 wherein said second antibody is the monoclonal antibody of Claim 37.

62. The kit of Claim 56 wherein said second antibody is the monoclonal antibody of Claim 40.

63. A murine hybridoma cell line capable of producing a monoclonal antibody which is immunologically reactive iwth shared determinants of an endotoxin or endotoxin-like lipopolysaccharide.

64. The cell line according to Claim 63 selected from the group consisting of cell line Re-1 (ATCC. No. HB 8482), Re-2 (ATCC. No. HB8481), LA-4 (ATCC. No. HB 8480), LA-5 (ATCC. No. HB 8479) and C-2 (ATCC. No. HB 8478).

INTERNATIONAL SEARCH REPORT

International Application No PCT/US84/02022

I. CLASSIFICATI N OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

US - 435/7, 810, 948; 260/112B

IPC-3 G01N 33/54; A61K 39/395

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	435/7, 18, 21, 34, 35, 39, 68, 172.2, 240, 810, 948 436/548,511, 807 260/112B, 112R 935/95,102, 103, 106, 110

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [5]

MEDLINE 1966-1984
Biosis Previews -1969-1985
Chemical Abstracts -1967-1984

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	N, Science, 220, issued 17 June 1983, Nurminen et al, 1279-1281.	1-5, 7, 13-18,20, 21, 43-46, 50-52
X	N, Journal of Immunological Methods, 43, issued 1981, Stevens et al, 199-207	1-5, 7, 13, 16-18, 20, 43, 44, 46
X	N, Infection and Immunity, 34(3), issued December 1981, Apicella et al, 751-756	1-5, 7-11, 13-18, 20-22, 43-46,50-52, 63
X	N, European Journal of Immunology, 12, issued 1982, Hiernaux et al. 797-803	1-5, 7-11, 13-18,20-22, 43-46, 50-52, 63
X	N, The Journal of Immunology, 115(4), issued October 1975, Caldwell et al, 963-968	1-5

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
14 January 1985	18 JAN 1985
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Esther M. Kepplinger ESTHER M. KEPPLINGER

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No 18
Y	N, Science, 220, issued 17 June 1983, Nurminen et al, 1279-1281	8-12, 19, 22-42,47-49,53-64
Y	N, Journal of Immunological Methods, 43, issued 1981, Stevens et al, 199-207	19,22-39,47 48, 53, 54, 56-61, 63, 64
Y	N, European Journal of Immunology, 12, issued 1982, Hiernaux et al, 797-803	19, 22-39, 47 48, 53, 54, 56-61, 63,64
Y	N, The Journal of Immunology, 115(4) issued October 1975, Caldwell et al, 963-968	40-42, 49, 55, 62-64
Y,P	N, The Journal of Infections Diseases, 149(4), issued April 1984, Puolakkainen et al, 598-604	1-64
Y,P	N, Infection And Immunity, 44(2), issued May 1984, Caldwell et al, 306-314	1-64
Y,	N, Infection And Immunity, 45(3), issued September 1984, Mutharia et al, 631-636	1-64

FURTHER INF RMATION CONTINUED FROM THE SECOND SHEET

X	N, Infection and Immunity, 42(3), issued December 1983, Sugasawara et al, 863-868	1, 3-5, 8, 9, 11, 13- 18, 21, 22, 43, 44, 46, 50, 51, 63
X	US, A. 4,115,543, Published 19 September 1978 See Column 4, lines 11-13, Wallace	1, 3, 4, 13
Y	US, A, 4,376,110, Published 08 March 1983, David	1-64

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.